Developmental Cell, Vol. 9, 675–685, November, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.devcel.2005.09.015

Efficient Protein Trafficking Requires Trailer Hitch, a Component of a Ribonucleoprotein Complex Localized to the ER in *Drosophila*

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Summary

Translational control of localized messenger mRNAs (mRNAs) is critical for cell polarity, synaptic plasticity, and embryonic patterning. While progress has been made in identifying localization factors and translational regulators, it is unclear how broad a role they play in regulating basic cellular processes. We have identified *Drosophila trailer hitch* (*tral*) as a gene that is required for the proper secretion of the dorsal-ventral patterning factor Gurken, as well as the vitellogenin receptor Yolkless. Surprisingly, biochemical purification of Tral revealed that it is part of a large RNA-protein complex that includes the translation/localization factors Me31B and Cup as well as the mRNAs for endoplasmic reticulum (ER) exit site components. This complex is localized to subdomains of the ER that border ER exit sites. Furthermore, *tral* is required for normal ER exit site formation. These findings raise exciting new possibilities for how the mRNA localization machinery could interface with the classical secretory pathway to promote efficient protein trafficking in the cell.

Introduction

Localization of mRNAs is used by many polarized cells as a means of restricting the distribution of a protein to a particular cytoplasmic domain. This mechanism for protein targeting within the cytoplasm is critical for embryonic patterning, synapse formation, and cell migration (Bashirullah et al., 1998; Kloc et al., 2002). Three methods of localization have previously been described. Active transport of mRNA along either microtubules or actin filaments has been directly demonstrated for a number of transcripts, suggesting a role for cytoskeletal motor proteins in mRNA localization (Bertrand et al., 1998; Cha et al., 2001; Fusco et al., 2003). Diffusion trapping involves the creation of a binding site for the message of interest at a particular subcellular location and allowing that site to passively trap target messages that it contacts by diffusion. Such a mechanism is used to localize *nanos* mRNA to the posterior pole of the developing *Drosophila* oocyte (Forrest and Gavis, 2003; Glotzer et al., 1997). Degradation protection involves selective stabilization of messages at the correct location while unlocalized messages are degraded. This type of mechanism is responsible for the posterior localization of a number of maternal messages during early embryogenesis in *Drosophila* (Bashirullah et al., 2001).

One of the most extensively characterized systems for studying mRNA localization is the *Drosophila* oocyte. Oocytes develop as part of an egg chamber, which is composed of the oocyte and 15 nurse cells surrounded by a layer of somatic follicle cells. The oocyte is connected to the nurse cells by a network of cytoplasmic bridges called ring canals (Spradling, 1993). Various mRNAs that are required for early embryogenesis are synthesized in the nurse cells and transported into the oocyte where some, such as *oskar* (*osk*) and *gurken* (*grk*), are localized to discrete subcellular locations (Ephrussi et al., 1991; Kim-Ha et al., 1991; Neuman-Silberberg and Schupbach, 1993).

While genetic and biochemical approaches have identified an ever increasing group of proteins required for mRNA localization during *Drosophila* oogenesis, recent work in *Drosophila* and other systems has revealed unexpected connections between the factors that regulate mRNA localization, mRNA stability, and translation. For instance, Cup was identified as an eIF4E binding protein that is required to both translationally repress *osk* mRNA as well as to recruit the localization factor Barentsz (Nakamura et al., 2004; Wilhelm et al., 2003). Similarly, the RNA helicase *me31B* has been shown to be required for translational repression of *osk* and *Bicaudal-D* in *Drosophila*, while its orthologs in yeast and humans are required for mRNA degradation (Coller et al., 2001; Cougot et al., 2004; Nakamura et al., 2001). Most surprisingly, *staufen*, which is the archetypal mRNA localization factor in *Drosophila* and vertebrates, has recently been shown to regulate message stability via the same components that control nonsense-mediated mRNA decay (Kim et al., 2005; St Johnston et al., 1991; Tang et al., 2001). Consistent with these factors affecting multiple aspects of mRNA metabolism, the *Drosophila* proteins required for localization and translational control have long been known to reside in large cytoplasmic particles that are both static and exhibit microtubule-dependent transport (Theurkauf and Hazelrigg, 1998). Furthermore, the yeast and human orthologs of one component of these particles, the translational repressor Me31B, have also been shown to be critical for the function of processing bodies (P bodies), large cytoplasmic particles that contain many of the regulators of mRNA stability (Cougot et al., 2004; Sheth and Parker, 2003). These results have suggested a model in which mRNA localization, translational regulation, and stability are integrated functions of the P body (Coller and Parker, 2004).

Because early patterning events in oogenesis are intimately connected to the proper regulation of cytoplasmic mRNA processing (i.e., translation, localization, and stability), we screened existing insertional mutants for defects in patterning to identify novel P body components. This screen identified a *Drosophila* gene, *trailer hitch* (*tral*), that is required for efficient secretion of the TGF-α family member Grk and the vitellogenin

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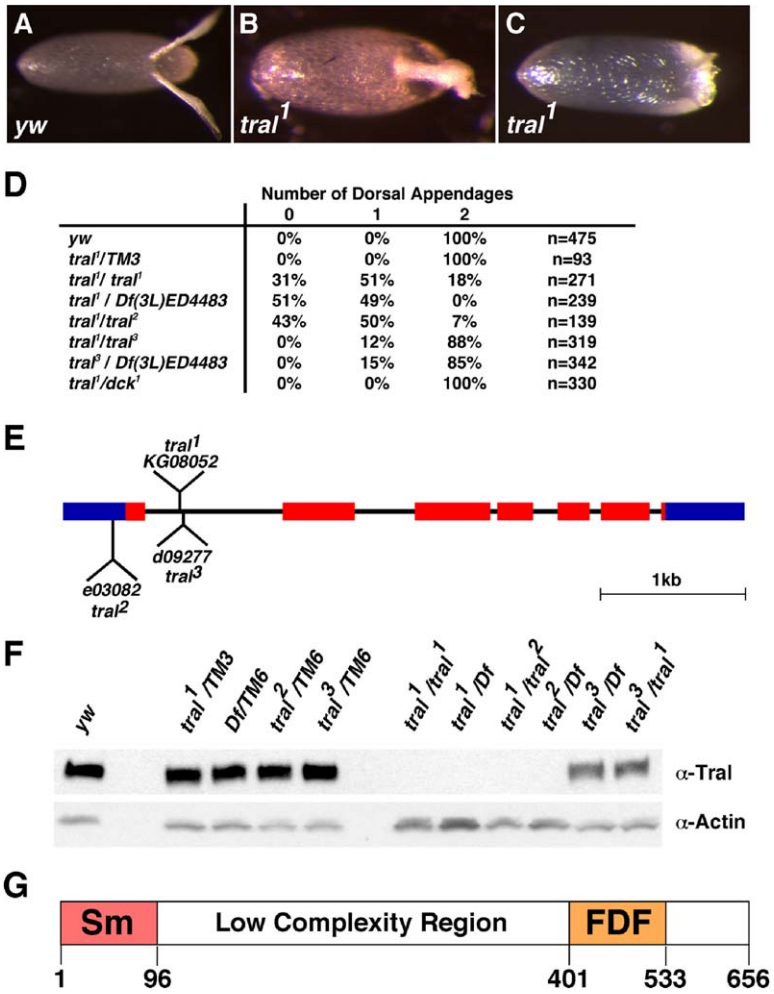

Figure 1. Characterization of the *tral* Locus

tral mutants cause ventralization of the eggshell.

(A–C) Two individualized dorsal appendages are present on eggs from *yw* females. *tral*[1] females lay ventralized eggs that either have (B) one fused dorsal appendage or (C) no dorsal appendages.

(D) Table of *tral* allelic series.

(E) Genomic organization of the *tral* locus. The open reading frame is marked in red, the untranslated regions are marked in blue, and the introns are marked in black. The insertion sites of the three *tral* alleles are indicated.

(F) Immunoblot for Tral and actin of ovarian extract from *tral* mutant females.

(G) Structure of the Tral protein. The Sm-related domain is marked in red and the FDF motif is marked in orange. These two domains are linked by a region of low sequence complexity composed primarily of glycine and asparagine.

receptor Yolkless (Yl). Our biochemical and genetic analysis of Tral revealed an unexpected connection between cytoplasmic mRNA processing events and trafficking through the secretory pathway.

Results

Tral Is Required for Proper Dorsal-Ventral Patterning

While screening P element insertions generated by the Berkeley *Drosophila* Genome Project (BDGP) gene disruption project for uncharacterized genes required for embryonic axis formation, we identified a female sterile P element insertion, KG08052, that exhibited defects in the dorsal-ventral patterning of the eggshell (Figures 1A–1D) (Bellen et al., 2004). The KG08052 insertion site lies within the first intron of CG10686, suggesting that disruption of this gene, which we have named *trailer hitch* (*tral*), is responsible for the dorsal-ventral patterning defect (Figure 1E). Quantitation of the dorsal-ventral patterning defect in eggs laid by females homozygous for the KG08052 insertion (*tral*[1]) revealed that 80% of eggs have either no dorsal appendages or display a single fused appendage—a phenotype indicative of ventralization of the eggshell (Figures 1A–1D). Females hemizygous for *tral*[1] showed an enhancement of the dorsal appendage phenotype, with 100% of eggs showing either 0 or 1 dorsal appendages, indicating that *tral*[1] is a strong hypomorphic allele.

In order to further characterize the *tral* locus, we obtained two additional insertions in the *tral* locus: e03082 (*tral*[2]), a PiggyBAC transposon insertion in the 5′UTR of *tral* and d09277 (*tral*[3]), a P element insertion in the first intron of *tral*. Ninety-three percent of eggs laid by *tral*[1]/*tral*[2] mothers display a ventralized eggshell phenotype (Figure 1D) consistent with *tral*[2] being a strong hypomorphic mutation. In contrast, while *tral*[3]/*tral*[1] mothers are sterile, only 12% of their eggs had ventralized eggshells, indicating that *tral*[3] is a weak hypomorphic allele of *tral* (Figure 1D). Because the *tral* locus is quite close to the *citron kinase* gene (*dck*), we performed complementation tests between *tral* and *dck* to determine whether our phenotype was due to the insertions affecting both genes. The lethal allele, *dck*[1], fully complements *tral*[1], indicating that eggshell ventralization in *tral* mutants is not due to disruption of *dck*. Thus, *tral*[1], *tral*[2], and *tral*[3] constitute an allelic series with respect to the strength of the ventralization phenotype and do not disrupt the closest neighboring gene, *dck*.

To confirm that *tral*[1], *tral*[2], and *tral*[3] disrupt Tral ex-

pression, we raised antibodies to the first 130 amino acids of Tral and probed immunoblots of ovaries derived from various *tral* allelic combinations for Tral protein. *tral*^{1} and *tral*^{2} in combination with either each other or with the deficiency *Df(3L)ED4483* decreased *tral* expression to an undetectable level, consistent with the disruption of *tral* expression being responsible for the observed ventralization of the eggshell (Figure 1F). Immunoblots of ovaries from *tral*^{3}/*tral*^{1} or *tral*^{3}/*Df(3L)ED4483* females showed a decrease in Tral protein expression as compared to *tral*^{3} heterozygotes or a *yw* control but did not completely eliminate expression, consistent with *tral*^{3} being a weak hypomorphic allele of *tral*. Although rescue of the mutant phenotype would be necessary to rule out the possibility that a second site mutation is the cause of the observed phenotypes, the fact that *tral*^{1}, *tral*^{2}, and *tral*^{3} constitute an allelic series with respect to both strength of phenotype and expression of *tral* strongly argues that the observed phenotypes are due to decreases in *tral* expression.

Tral Is Required for Efficient Trafficking of Grk Protein

One of the key events in dorsal-ventral patterning is the localization of *grk* mRNA to the dorsal-anterior region. The localization of *grk* mRNA in turn causes the trafficking of Grk protein to be confined to dorsal-anterior endoplasmic reticulum (ER)-Golgi units. It is this localized secretion of Grk that instructs the dorsal follicle cells to assume a dorsal cell fate (Neuman-Silberberg and Schupbach, 1993, 1996). These dorsal follicle cells then secrete the proper eggshell components to generate a dorsal appendage (Waring, 2000). The dorsal-ventral patterning defect of the *tral* mutants suggested that *tral* might regulate some aspect of the localization or secretion of Grk. In wild-type egg chambers, Grk protein is expressed homogeneously throughout the oocyte during stages 6–7 and then is only found in small puncta near the plasma membrane in the dorsal-anterior region of the oocyte during stages 8–10 (Figures 2B and 2C). These small Grk puncta are known to coincide with sites of exit from the ER (Herpers and Rabouille, 2004). In both *tral*^{1} homozygotes and *tral*^{1} hemizygotes, we observe abnormally large Grk puncta in 48% (n = 50) of homozygotes and 63% (n = 52) of hemizygotes during stages 6–8 (Figures 2E and 2F; see Figure S1 in the Supplemental Data available with this article online). This suggests that mutations in *tral* disrupt some aspect of Grk trafficking through the secretory pathway.

Conceivably, *tral* mutants could affect Grk trafficking either by interfering with the proper localization/translational control of the *grk* message, by disrupting the microtubule cytoskeleton, or by blocking the normal trafficking of Grk protein through the secretory pathway. To test these possibilities, we first assayed the localization of *grk* mRNA in *tral* mutant egg chambers by in situ hybridization. The localization of *grk* mRNA to the dorsal-anterior region of the oocyte during stages 8–10 is normal in *tral* mutant egg chambers (Figures 2G and 2H). This result argues against defects in *grk* mRNA localization being responsible for the Grk trafficking defect observed in *tral* mutants.

The fact that *grk* mRNA is correctly localized argues that the normal polarity of the microtubule cytoskeleton is intact in *tral* mutants, as a polarized microtubule network is essential for *grk* mRNA localization (MacDougall et al., 2003). To confirm that the microtubule polarity is intact, we also examined whether the localization of Osk protein to the posterior is normal in *tral* mutant egg chambers. Because the correct localization of Osk protein to the posterior requires both normal microtubule polarity and the proper localization of *osk* mRNA, this assay should reveal any functional defects in either the microtubule polarity or the transport of *osk* mRNA. Whereas large Grk puncta accumulate in the oocytes of *tral* mutants, Osk protein is present at the posterior of the oocyte (Figures S2A and S2B). Consistent with this result, mutations in *tral* do not affect the normal anterior-posterior gradient of microtubule density in stage 9 egg chambers (Figures S2C and S2D). Thus, mutations in *tral* do not affect either microtubule polarity or the transport of the *grk* and *osk* messages. This result may seem paradoxical, as *grk* signaling early in oogenesis is required to establish the microtubule polarity of the oocyte (Roth et al., 1995). However, the establishment of microtubule polarity is less sensitive to changes in the level of *grk* signaling than dorsal appendage formation. Because none of our *tral* alleles cause complete ventralization of the eggshell, it is not surprising that we have been able to selectively affect dorsal appendage formation without altering the microtubule polarity of the oocyte.

To rule out that large Grk puncta are due to a defect in Grk translational control, we examined the distribution of large Grk puncta during stages 8–10. If there was a defect in translational repression of *grk* mRNA, Grk protein should accumulate broadly throughout the oocyte. While some large Grk puncta are mislocalized to the side of the nucleus facing away from the oocyte cortex, both normal sized and large Grk puncta are restricted to the dorsal-anterior region of the oocyte (Figures 2E, 2F, and 3E; Figures S1A–S1F, S2A, and S2B). Because a defect in translational control would be expected to yield high levels of Grk protein throughout the oocyte, this result argues that the large Grk foci are not due to a loss of translational repression of the *grk* message. Because the polarity of the microtubule cytoskeleton and the localization/translation of *grk* mRNA appear normal in *tral* mutant egg chambers, we next tested the hypothesis that the formation of large Grk puncta in *tral* mutants is due to a defect in the trafficking of Grk.

In a variety of systems, ER exit sites are closely associated with Golgi units, presumably due to the role of ER trafficking in establishing and maintaining the Golgi (Bevis et al., 2002; daSilva et al., 2004). Because previous work established that small Grk puncta are coincident with ER exit sites, also known as the transitional ER, we examined the effects of *tral* mutants on the distribution of Grk and its association with the Golgi (Herpers and Rabouille, 2004). In wild-type egg chambers, the majority of Grk protein is present in small puncta that are closely associated with an individual Golgi complex that is positive for the Golgi marker Lava lamp (Figures 2I–2K). However, in *tral* mutants, the large Grk puncta have lost their intimate association with the

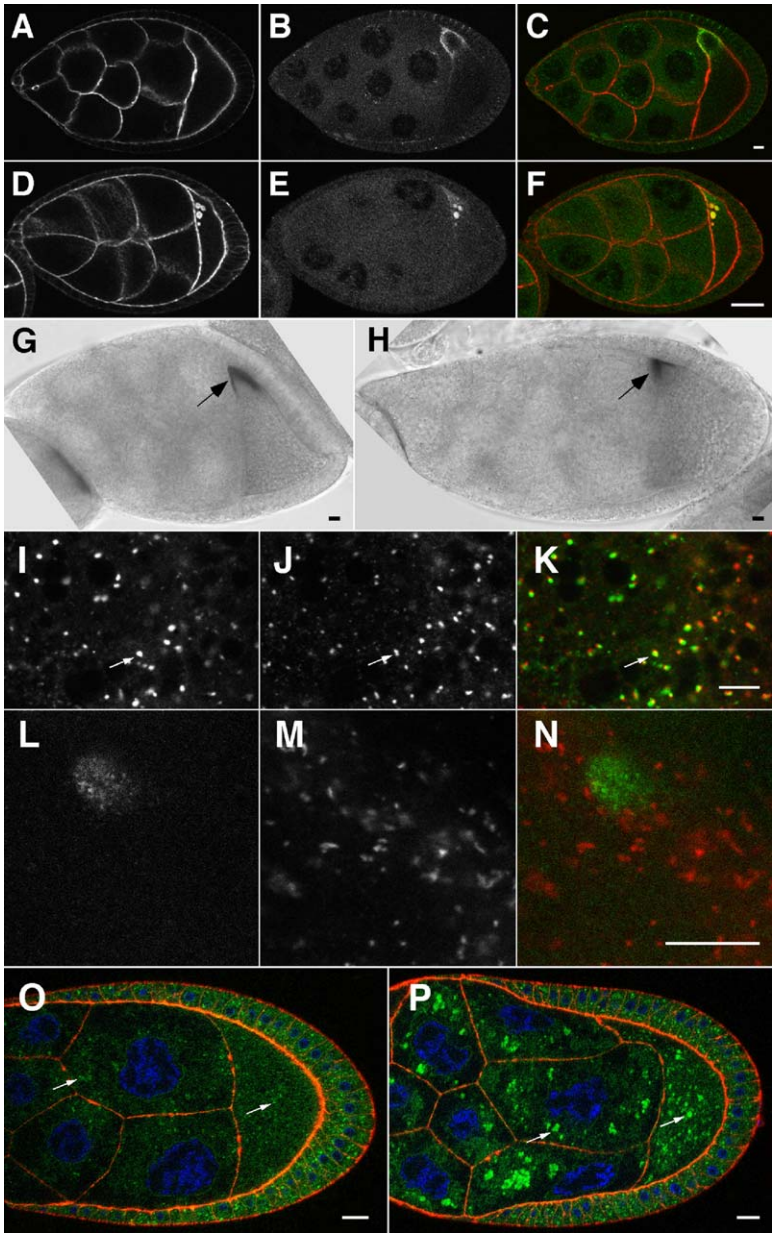

Figure 2. *tral* Mutants Cause Grk Protein to Accumulate in Large Puncta and Alter ER Exit Site Morphology

(A–F) Stage 9 *yw* egg chamber stained for (A) actin and (B) Grk; (C) merged image. Stage 8 *tral*^{1} homozygous egg chamber stained for (D) actin and (E) Grk; (F) merged image. Note the large puncta that contain Grk. Actin is in red. Grk is in green.

(G and H) *grk* mRNA is localized to the dorsal-anterior region of the oocyte (arrows) in both (G) *yw* and (H) *tral*^{1} homozygous egg chambers. Grk puncta and Golgi units are paired together in *yw* egg chambers (arrows).

(I–K) Shown is a glancing section above the nucleus of a stage 9 *yw* egg chamber stained for (I) Grk and (J) Lava lamp; (K) merged image. Large Grk puncta are morphologically similar to regions of the ER lacking exit sites and are not associated with the Golgi.

(L–N) Shown is a section through a large Grk focus in a *tral*^{1}*/Df(3L)ED4483* egg chamber stained for (L) Grk and (M) Lava lamp; (N) merged image. Lava lamp is in red. Grk is in green.

(O) ER exit sites marked with Sar1-GFP form small foci distributed throughout a stage 9 *yw* egg chamber (arrows).

(P) ER exit sites marked with Sar1-GFP form abnormally large foci throughout a stage 9 *tral*^{1} *Sar1-GFP/Df(3L)ED4483* egg chamber (arrows). Sar1-GFP is in green. Actin is in red. Nuclei stained with DAPI are in blue.

The scale bar represents 10 μm.

Golgi (Figures 2L–2N). This suggested that the formation of large Grk foci might be due to a defect in ER exit.

The COPII complex, which is required for ER-to-Golgi trafficking, is known to label discrete sites on the ER (Bevis et al., 2002; Hobman et al., 1998). Furthermore, a number of experiments have implicated these COPII sites and the regions surrounding them in exit from the ER (Bevis et al., 2002; Mironov et al., 2003). Using GFP-Sar1 as a marker for COPII complex formation, we examined the distribution of ER exit sites in wild-type and *tral* hemizygous egg chambers. GFP-Sar1 is distributed in small puncta throughout the nurse cells and oocyte in wild-type egg chambers (Figure 2O). However, this organization is severely disrupted in *tral*^{1}*/Df(3L)ED4483* egg chambers. In these egg chambers, the GFP-Sar1 is found in abnormally large puncta similar to those observed for Grk protein (Figure 2P). Thus, *tral* is required for normal ER exit site distribution and morphology. The accumulation of Grk in large foci that are not correctly associated with the Golgi, together with the role of *tral* in organizing ER exit sites, argues that the disruption of ER exit sites in *tral* mutants leads to a functional defect in ER-Golgi trafficking. It is this disruption of ER-Golgi trafficking that likely underlies the failure in dorsal-ventral patterning observed in *tral* mutants.

tral Is Required for Normal Trafficking of the Vitellogenin Receptor Yl

If *tral* plays a general role in ER exit site function, one would expect to observe defects in the trafficking of other secreted proteins. In order to test this, we next assayed the effects of *tral* mutants on the trafficking of

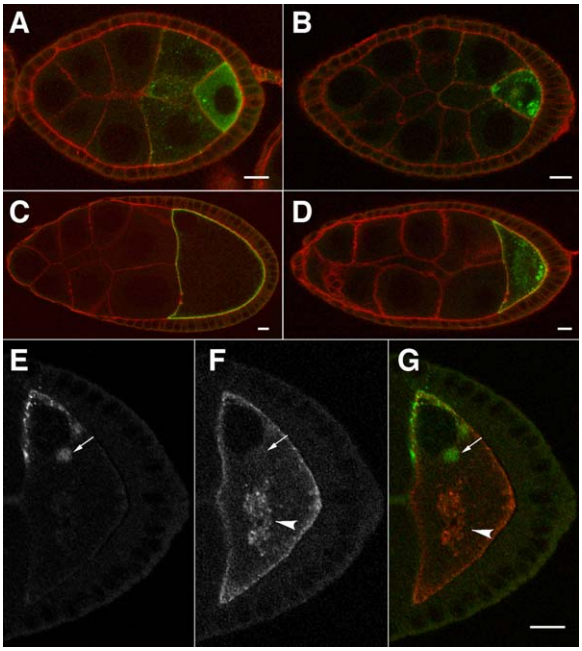

Figure 3. Yl Trafficking Is Disrupted in *tral* Mutant Egg Chambers

(A) Yl protein (green) is homogeneously distributed in the oocyte of stage 7 *yw* egg chambers.

(B) Yl protein (green) accumulates in large puncta that do not label with actin (red) in stage 7 *tral*^{1}/*Df(3L)ED4483* egg chambers.

(C) Yl protein (green) is homogeneously distributed on the plasma membrane of stage 9 *yw* egg chambers.

(D) Yl protein (green) accumulates in large puncta that do not label with actin (red) in stage 9 *tral*^{1}/*Df(3L)ED4483* egg chambers.

(E–G) Grk and Yl puncta are not identical. A stage 8 *tral*^{1}/*Df(3L)ED4483* egg chamber labeled for (E) Grk and (F) Yl; (G) merged image. Grk is in green and Yl is in red in the merged image. The scale bar represents 10 μm.

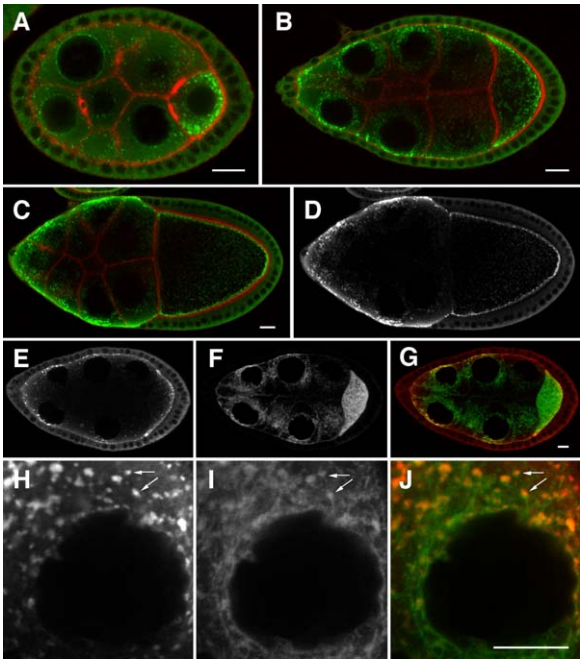

Figure 4. Tral Protein Is Dynamically Localized during Oogenesis and Is Associated with the Endoplasmic Reticulum

(A) Tral protein (green) is concentrated at the posterior of the developing oocyte in stages 1–6 (stage 6 is shown; actin is in red).

(B) Tral is transiently enriched at the anterior of the oocyte during stages 7 and 8 (stage 8 is shown).

(C) Tral accumulates at the posterior pole during stages 9 and 10 with a lower level of staining all along the oocyte cortex (stage 9 is shown).

(D) The cortical staining is most easily seen in the separated Tral channel.

(E–G) Tral colocalizes with the ER in oocytes and nurse cells (stage 8 is shown). (E) Tral. (F) GFP-KDEL. (G) Merged image.

(H–J) Tral particles are associated with discrete subdomains of the ER in nurse cells (arrows). (H) Tral. (I) GFP-KDEL. (J) Merged image. The scale bar represents 10 μm.

the vitellogenin receptor Yl. Previous work on Yl has shown that in wild-type egg chambers, Yl protein is distributed homogeneously throughout the ER of the oocyte with an occasional small puncta until stage 8, when all of the Yl protein is transported to the plasma membrane (Schonbaum et al., 2000) (Figures 3A and 3C). Homozygous *tral*^{1} oocytes showed no obvious disruption of Yl trafficking (data not shown). However, 75% (n = 55) of hemizygous *tral*^{1} oocytes showed Yl foci within the oocyte during stages 6–9 (Figures 3B and 3D). Therefore, *tral* is required for the trafficking of proteins besides Grk and likely plays a general role in promoting exit from the ER.

We next asked whether the Grk and Yl foci are distinct in *tral*^{1} hemizygous oocytes. Immunostaining for both Grk and Yl revealed that the large foci for each protein are separate (Figures 3E–3G). This suggests that the two proteins use separate trafficking pathways that both require *tral*. Our observation that the trafficking of Grk is more sensitive to decreases in *tral* function than the trafficking of Yl is consistent with this idea.

Tral Is Localized to Discrete Sites on the Endoplasmic Reticulum

In order to better understand where Tral might be acting during oogenesis, we examined the distribution of Tral in the egg chamber. Within the nurse cells, Tral was present in discrete particles throughout oogenesis (Figures 4A–4C). However, within the oocyte, Tral showed a dynamic distribution—first being localized to the posterior of the oocyte during stages 1–6 (Figure 4A; Figures S3A and S3B), accumulating briefly at the anterior during stages 7–8 (Figure 4B; Figures S3C and S3D), followed by weak accumulation along the oocyte cortex with substantial enrichment at the posterior pole during stages 9–10 (Figures 4C and 4D; Figures S3E and S3F).

Live imaging of GFP-Tral in nurse cells demonstrated that while there are a number of motile Tral particles, a substantial fraction of the particles are immobile and appear to be tethered to a large reticular structure reminiscent of the ER (Movie S1). This result, together with the finding that *tral* mutations interfere with the ER-Golgi trafficking of Grk and Yl, suggested that Tral might function on the surface of the ER. To test this, we visualized the ER by using a transgenic *Drosophila* line that expresses GFP fused to the KDEL ER retention signal (GFP-KDEL) only in the germline. While we find

that Tral protein colocalizes with the ER of the oocyte, the dense accumulation of ER membranes within the oocyte made close examination of the sites of colocalization difficult (Figures 4E–4G; data not shown). We therefore focused our analysis on Tral particles within the nurse cells where ER membranes do not completely fill the cytoplasm. Immunostaining for Tral and GFP-KDEL revealed that the numerous Tral particles within the nurse cells are all associated with subdomains of the ER (Figures 4H–4J). These subdomains are typically sites of concentrated ER and are often present at the end of ER tubules (Figures 4H–4J). Given the Grk and Yl secretion defects observed in *tral* mutants, the localization of Tral to discrete domains of the ER suggests that Tral acts directly to regulate ER exit site function.

Sequence Analysis of the Trailer Hitch Protein Family Suggests a Role in Cytoplasmic mRNA Processing

Sequence analysis of *tral* revealed that it is a member of a highly conserved family of proteins that is present in virtually all eukaryotes (Albrecht and Lengauer, 2004; Anantharaman and Aravind, 2004). The Trailer Hitch family of proteins contains several conserved sequence features including an amino-terminal Sm-related domain and a carboxy-terminal FDF motif (Figure 1G) (Albrecht and Lengauer, 2004; Anantharaman and Aravind, 2004). The canonical Sm domain is a motif common to a number of proteins that regulate various aspects of RNA metabolism, including splicing and mRNA stability (Bouveret et al., 2000; Mayes et al., 1999; Tharun et al., 2000). Thus, the identification of a divergent Sm domain within *tral* suggests that it may regulate some aspect of RNA metabolism. The identification of an FDF motif in *tral* also supports a role for *tral* in posttranscriptional gene regulation. The FDF motif was originally defined as a motif conserved within the EDC3 family of proteins (Anantharaman and Aravind, 2004). EDC3p in *Drosophila* has been shown to promote mRNA decay by enhancing removal of the 5′ cap, indicating that the FDF motif is also likely to play a role in RNA recognition or stability (Kshirsagar and Parker, 2004). In addition to the sequence analysis, the newt ortholog of *tral*, Rap55, has been shown to be part of a cytoplasmic RNA-protein complex (Lieb et al., 1998). Together, these observations suggest that the Trailer Hitch family may regulate some aspect of RNA metabolism, such as stability, localization, or translation.

Tral Is a Component of an RNA-Protein Complex Associated with the Endoplasmic Reticulum

In order to better understand the role of Tral in regulating membrane trafficking, we sought to identify Tral-associated proteins by immunoprecipitating Tral from *Drosophila* embryo extract using our Tral antibody. By colloidal blue staining, we found three major bands that specifically coimmunoprecipitated with Tral and not with an IgG control: p147, p70, and p50 (Figure 5A). Using mass spectrometry, we identified p147 as the eIF4E binding protein Cup, and p70 as poly(A) binding protein (PABP). p50 was found to be a mixture of the RNA binding protein Ypsilon Schactel (Yps) and the RNA helicase Me31B. To confirm the identities of the Tral-associated proteins, we immunoprecipitated Tral from ovarian extracts and immunoblotted for Cup, Yps, and Me31B (Figure 5B). Me31B, Yps, and Cup all specifically coimmunoprecipitate with Tral, indicating that these proteins are bona fide components of the complex. Because Me31B, Yps, and Cup have been previously shown to be part of an RNA-protein complex, we tested the ability of each protein to coimmunoprecipitate with Tral in RNase-treated ovarian extracts (Nakamura et al., 2004; Wilhelm et al., 2003). We found that while the association of Tral with Me31B, Yps, and Cup is RNase resistant, the association of Yps with Cup is sensitive to RNase treatment, indicating the presence of RNA in the complex (Figure 5B).

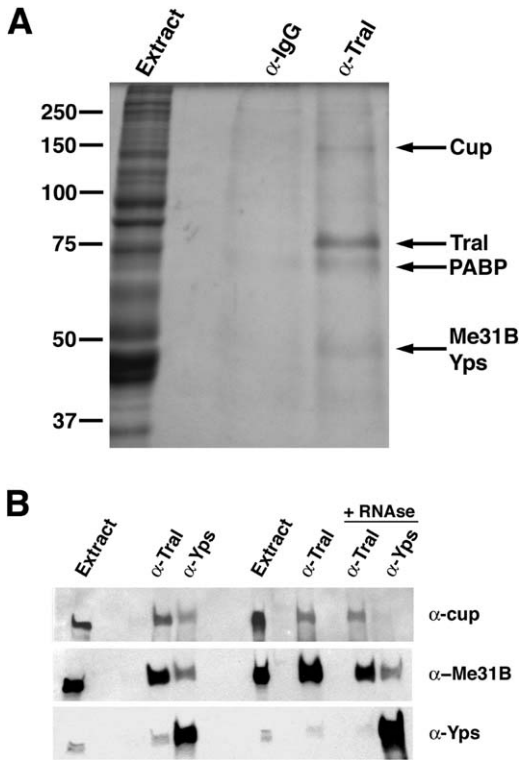

Figure 5. Tral Is Part of a Large RNA-Protein Complex

(A) Immunoprecipitations of embryo extracts with either α-Tral antibodies or rabbit IgG. Tral and four additional polypeptides (arrows) coimmunoprecipitate with α-Tral antibodies, but not with the rabbit IgG control. Identification of polypeptides is described in Experimental Procedures. Migration of molecular weight standards by SDS-PAGE is indicated on the left.

(B) Immunoblot for Cup, Me31B, and Yps in immunoprecipitates from ovarian extract using α-Tral, α-Yps, or rabbit IgG antibodies. RNase treatment of immunoprecipitates using either α-Tral or α-Yps shows that the association of Tral with Cup, Me31B, and Yps is resistant to RNase treatment. However, the association of Yps with Cup is sensitive to RNase treatment, indicating the presence of RNA in the complex.

Previous work has shown that Me31B, Cup, and Yps colocalize in vivo (Nakamura et al., 2004; Wilhelm et al., 2003). In order to demonstrate that Tral is part of the Me31B-Cup-Yps complex in vivo, we immunostained egg chambers for Tral and Me31B as well as Tral and Cup. The particulate staining in nurse cells showed a high degree of overlap for both the Tral/Cup (Figures 6A–6C) and Tral/Me31B (Figures 6D–6F) double-labeled

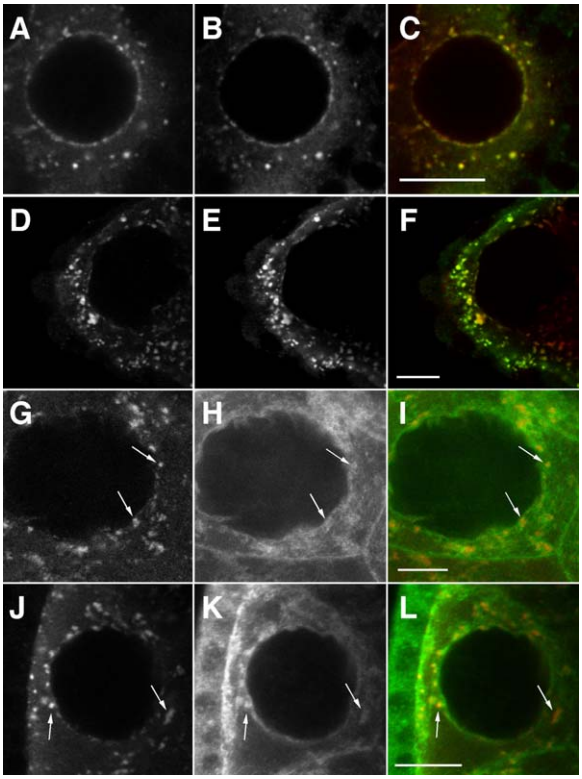

Figure 6. Me31B and Cup Colocalize with Both Tral and the ER in Egg Chambers

Tral and Cup colocalize to the same particles in nurse cells.
(A–C) (A) Tral. (B) Cup. (C) Merged image.
(D–F) Tral and Me31B colocalize to the same particles in nurse cells. (D) Tral. (E) Me31B. (F) Merged image.
(G–I) Me31B particles localize to discrete sites on the ER (arrows). (G) Me31B. (H) GFP-KDEL. (I) Merged image.
(J–L) Cup particles localize to discrete sites on the ER (arrows). (J) Cup. (K) GFP-KDEL. (L) Merged image.
The scale bar represents 10 μm.

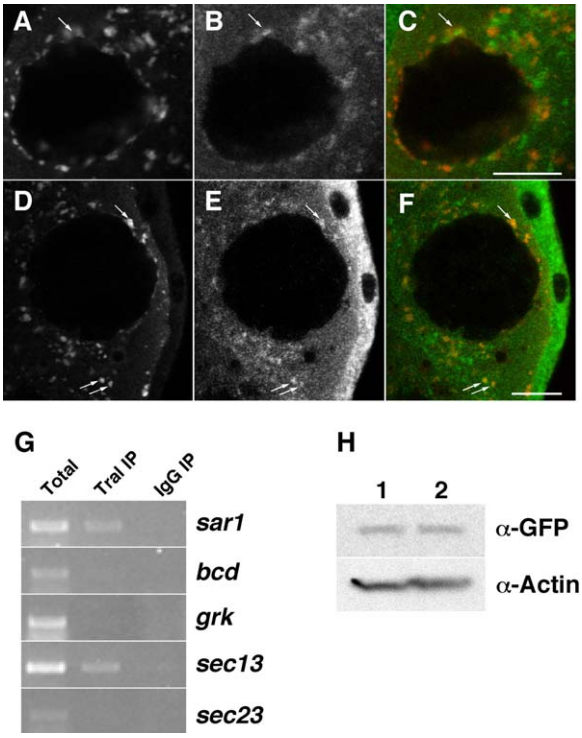

Figure 7. Components of the Tral Complex Neighbor ER Exit Sites and Are Associated with mRNAs Encoding ER Exit Site Proteins

(A–C) Cup localizes to sites on the ER that border and overlap SAR1 sites (arrow). (A) Cup. (B) GFP-Sar1. (C) Merged image.
(D–F) Me31B localizes to sites on the ER that border and overlap SAR1 sites (arrows). (D) Me31B. (E) GFP-Sar1. (F) Merged image. The scale bar represents 10 μm.
(G) *sar1* and *sec13* mRNAs are enriched in Tral immunoprecipitates while *bcd*, *grk*, and *sec23* mRNAs are not.
(H) Mutations in *tral* do not affect the expression of Sar1-GFP protein. Lane 1: ovarian extract from *tral*^{1} *Sar1-GFP/TM3*. Lane 2: ovarian extract from *tral*^{1} *Sar1-GFP/Df(3L)ED4483*.

egg chambers. Furthermore, the temporal-spatial pattern of Tral localization within the oocyte is identical to that previously described for Cup, Me31B, and Yps (Figures 4A–4C; Figure S3) (Nakamura et al., 2001, 2004; Wilhelm et al., 2000, 2003). These results, together with the previously demonstrated colocalization of Me31B, Cup, and Yps, indicate that Tral, Cup, Me31B, and Yps all exist as a complex in vivo.

Because Tral is present on discrete domains of the ER, we next asked whether other components of the complex were also present on the ER. Colocalization studies of GFP-KDEL with either Me31B or Cup showed that Me31B (Figures 6G–6I) and Cup (Figures 6J–6L) are both present on discrete ER subdomains. This observation, together with the biochemical analysis of the Tral complex, demonstrates that Tral is part of an RNA-protein complex that is associated with the ER.

The Tral Complex Is Associated with a Subset of ER Exit Sites and Is Associated with *sar1* mRNA

Because mutations in *tral* have such striking effects on morphology of COPII foci, we sought to define the relationship between these foci and components of the Tral complex. Using GFP-Sar1 as a marker for COPII complex formation, we found that while some COPII sites are not associated with the Tral complex, a number of sites either colocalize with or are bordered by the Tral complex (Figures 7A–7F). These observations were highly suggestive of a direct role in regulating exit site function, as recent work has implicated the regions around COPII sites in exit from the ER (Bevis et al., 2002; Mironov et al., 2003).

In order to explain how an RNA-protein complex might regulate ER exit site function, we hypothesized that Tral regulates the transcripts for COPII components on the surface of the ER. If this were true, then Tral complexes should contain the messages of COPII components. To test this hypothesis, we immunoprecipitated Tral from ovarian extracts, isolated RNA from the pellet, and assayed for the presence of a variety of transcripts by RT-PCR. This experiment showed that the transcripts for the COPII components *sar1* and *sec13* were enriched in Tral immunoprecipitates, while *bcd*, *grk*, and *sec23* messages were not (Figure 7G). Because Tral is biochemically associated with the *sar1* message and mutations in *tral* cause profound disrup-

tion of the distribution of Sar1 protein, *sar1* mRNA is a likely regulatory target of the Tral complex.

One trivial way that this regulation could occur is by general derepression of a maternal pool of the *sar1* message causing overexpression of Sar1 protein and the accumulation of large Sar1 foci. However, the levels of GFP-Sar1 are equivalent in *tral*^{1} heterozygotes and *tral*^{1} hemizygotes (Figure 7H). This argues against bulk changes in translation or stability of the *sar1* message and suggests that any regulation of the *sar1* message is likely to be restricted to a subset of the transcript pool.

Discussion

In this study, we used a genetic screen for genes required for normal axis formation to identify uncharacterized components of the P body. This screen identified *tral* as a gene that is required for dorsal-ventral polarity and whose protein copurifies and colocalizes with the P body marker Me31B, validating this approach. Surprisingly, we found that the P body/Tral complex is localized to discrete subdomains of the ER and that *tral* is required for efficient secretion of two proteins: Grk and Yl. These results have revealed an unexpected role for cytoplasmic mRNA processing in regulating protein trafficking through the secretory pathway.

The Role of *tral* in the Secretory Pathway

A number of findings support a direct role for *tral* in promoting efficient trafficking through the secretory pathway. First, *tral* is required for the efficient secretion of both Grk and Yl. Second, immunohistochemistry places the Tral complex on the ER in close association with a subset of ER exit sites in nurse cells, although further analysis at the EM level will be necessary to demonstrate this conclusively. Third, Tral protein is biochemically associated with the transcripts of two ER exit site components: *sar1* and *sec13*. Last, *tral* is required for normal distribution of Sar1 protein, arguing that the *sar1* message is a regulatory target of *tral*. Together, these results suggest that *tral*'s role in secretion is to regulate the transcripts of ER exit site components on the surface of the ER.

ER exit sites have traditionally been defined as localized foci of components of the COPII complex, which is required for ER exit (Bednarek et al., 1995; Bevis et al., 2002; Espenshade et al., 1995; Hobman et al., 1998). However, recent work has shown that proteins actually exit from the region of the ER surrounding these foci, rather than from the foci themselves (Mironov et al., 2003). Because these foci are quite stable, this result has led to a model where "ER exit sites" are actually storage sites to allow a high concentration of COPII components to be available to drive local vesicle formation.

This model fits quite nicely with our results. The observations that the Tral complex borders a subset of ER exit sites and is associated with *sar1* mRNA suggest that the Tral complex regulates *sar1* expression on the surface of the ER. Because recruitment of Sar1 is the first step in assembling the COPII complex on the membrane, Sar1 is an excellent target for regulating the size and distribution of ER exit sites (Aridor et al., 2001). Consistent with this, *tral* mutants display abnormalities in both ER exit site morphology and secretion. Thus, the properly regulated assembly of COPII foci/ER exit sites is critical for normal ER-to-Golgi trafficking.

Because many components of the secretory pathway are highly conserved, one might expect *tral* orthologs to also play a role in secretion. Genetic screens in *Drosophila* and *C. elegans* have suggested that this family of proteins does play a conserved role in the regulation of membrane trafficking. The yeast ortholog of *tral*, Scd6, was identified as a high copy suppressor of a deletion of the clathrin heavy chain locus, while the worm ortholog, *car-1*, was identified in RNAi screens for genes that are required for cytokinesis (Nelson and Lemmon, 1993; Zipperlen et al., 2001). While there have been no additional studies of Scd6 to determine whether it acts directly or indirectly in the secretory pathway, abnormalities in membrane trafficking to the late cleavage furrow have been found in *car-1*-depleted embryos (J. White, personal communication). Furthermore, it has also been found that disruption of *car-1* leads to ER morphology defects similar to the foci of Grk and Yl that we observe in the *Drosophila* oocyte (J. White, personal communication). Our findings that Tral is associated with the ER and is required for efficient trafficking of secreted proteins are consistent with the phenotypes described in *C. elegans* and suggest that the role of the *trailer hitch* family in regulating membrane trafficking is conserved.

Given these similarities, one might have anticipated that *tral* mutants would also have defects in cytokinesis. However, it is likely that none of our hypomorphic alleles of *tral* disrupts *tral* function sufficiently to display a cytokinesis defect. Consistent with this interpretation, it is clear that only small amounts of Tral protein are required for some of its functions. For instance, *tral*^{1} homozygotes display no defect in Yl trafficking, but *tral*^{1} hemizygotes do, even though *tral*^{1} homozygous or hemizygous ovaries express undetectable amounts of Tral. The identification of stronger alleles of *tral* will likely be necessary to determine whether the role of *tral* in cytokinesis is conserved between *Drosophila* and *C. elegans*.

ER Trafficking and Cytoplasmic mRNA Processing

One of the most unexpected results of our studies was the identification of Tral as a component of an RNA-protein complex that is required for efficient membrane trafficking. Because *cup* and *me31B* have been implicated in various aspects of mRNA localization, mRNA stability, and translational control, this finding provided an unexpected link between cytoplasmic mRNA processing and the secretory pathway. While previous studies of *cup* and *me31B* did not describe any defect in secretion, such a role would likely have been missed, as the strongest alleles of *cup* and *me31B* cause egg chamber degeneration before either Grk or Yl secretion could be examined (Keyes and Spradling, 1997; Nakamura et al., 2001, 2004; Wilhelm et al., 2003). Because the Tral complex localizes to the ER and *sar1* mRNA is associated with Tral, we have proposed that Tral promotes proper ER exit site formation by local regulation of *sar1* expression on the ER.

The idea of a link between ER function and cytoplasmic mRNA processing is consistent with recent work in a number of other systems. In rice endosperm, prolamine mRNA, which encodes a storage protein used to support growth of the developing embryo, is targeted to discrete domains of the endoplasmic reticulum via its 3′UTR (Choi et al., 2000). This targeting is believed to be necessary for the formation of a specialized protein storage body derived from the ER. In *Saccharomyces cerevisiae*, the mRNA for the membrane protein Ist2p is localized to the cortical ER of daughter cells and this localization event allows Ist2p to be transported to the plasma membrane independent of the classic secretory pathway (Juschke et al., 2004). Our work on *tral* suggests that the connection between cytoplasmic mRNA processing and ER function may be broader than these two isolated examples and that cytoplasmic mRNA processing regulates exit from the ER in addition to its previously established role in the translocation of prolamine and Ist2p into the ER. These findings raise exciting new possibilities for how cytoplasmic mRNA processing could interface with the classical secretory pathway to promote efficient protein trafficking in the cell.

Experimental Procedures

Drosophila Strains and Culture

Fly stocks were cultured at 22°C–25°C on standard food. The $tral^1$ allele and *Df(3L)4483* were obtained from the Bloomington Stock Center at Indiana University. The $tral^2$ and $tral^3$ alleles were obtained from the Exelixis *Drosophila* Stock Collection at Harvard Medical School (Thibault et al., 2004). The $y^1\ w^{67c23}$ strain is described in FlyBase. GFP-Sar1 and GFP-Tral protein traps were supplied by M.B. The dck^1 strain was obtained from Silvia Bonaccorsi (Naim et al., 2004). The KDEL-GFP line was supplied by Mary Lilly (Snapp et al., 2004).

Antibody Generation

The first 160 amino acids of Tral (Tral160) were each cloned into pGEX-2T and expressed as C-terminal fusions to glutathione S-transferase in *Escherichia coli*. Rabbit antisera were generated and affinity purified as previously described (Wilhelm et al., 2000). Affinity-purified α-Tral and crude Tral antisera behaved the same in all applications.

Extract Preparation

Embryo extracts were prepared by collecting either 0- to 4-hr or 0- to 17-hr embryos as previously described in Gunawardane et al. (2001) with the following modifications. Dechorionated embryos were resuspended 1:2 (w/v) with *Drosophila* extract buffer (DXB: 25 mM HEPES [pH 6.8], 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 250 mM sucrose) containing 10 μg/ml aprotinin, leupeptin, pepstatin, and 1 mM phenylmethanesulfonyl fluoride. The extracts were homogenized with five strokes in a 5 ml Dounce homogenizer, then centrifuged at 10,000 × g for 15 min at 4°C in a Beckman TLX ultracentrifuge (Fullerton, CA). The supernatant was recentrifuged and the supernatant was stored at −80°C.

Ovary extracts for protein analysis were prepared from approximately 300 hand-dissected ovaries. Ovaries were resuspended in 1 ml of DXB and homogenized in a 1 ml Dounce homogenizer with 10 strokes of the pestle.

Ovary extracts for RT-PCR experiments were prepared from approximately 200 μl of packed hand-dissected ovaries. Ovaries were resuspended in 2 ml of DXB and homogenized in a 5 ml Dounce homogenizer with 10 strokes of a tight-fitting Teflon-coated pestle and then centrifuged at 10,000 × g for 15 min at 4°C in a microfuge. The supernatant was collected and centrifuged a second time. The supernatant from the second spin was collected and used immediately for immunoprecipitations.

Immunoblots

Immunoblot analysis was performed as described in Wilhelm et al. (2000) with the following modifications. The following primary antibodies were used: 1:1000 dilution α-Cup rat antibody (Keyes and Spradling, 1997), 1:5000 α-Me31B rat antibody (Nakamura et al., 2001), 1:2000 α-Tral rabbit antibody, 1:2000 α-GFP rabbit antibody (Torrey Pines, Houston, TX), or 1:200 mouse α-actin antibody (JLA-20, Developmental Studies Hybridoma Bank, Iowa City, IA).

Immunoprecipitations of Yps and Tral and Identification of Tral-Associated Proteins

Immunoprecipitations were carried out as described in Wilhelm et al. (2000). p147, p70, and p50 were resolved by SDS-PAGE and in-gel digestion of proteins was performed essentially as described in Jiménez et al. (1998).

Immunostaining and Fluorescence Microscopy

Immunostaining was done as described in Wilhelm et al. (2003) with the following alterations. Ovaries were dissected in room temperature Grace's media and fixed for 10 min in one part 3.7% paraformaldehyde in PBS to six parts heptane. Primary antibodies were diluted as follows: 1:1000 rat α-Cup (Keyes and Spradling, 1997), 1:3000 rabbit α-Osk (Markussen et al., 1995), 1:2000 rabbit α-Tral, 1:200 rat α-Yl (Schonbaum et al., 2000), 1:5 mouse α-Grk (1D12, Developmental Studies Hybridoma Bank), 1:5000 rabbit α-Lava lamp (Sisson et al., 2000), 1:3000 goat α-GFP (Research Diagnostics, Concord, MA), and 1:2000 rabbit α-GFP (Torrey Pines). The following secondary antibodies were used: goat α-rabbit and α-rat AlexaFluor488, goat α-mouse Cy3, goat α-rat AlexaFluor568, donkey α-goat AlexaFluor488, donkey α-rat Cy3, and donkey α-rabbit Cy3.

Direct labeling of microtubules with 1:100 FITC mouse DM1 (Sigma, St. Louis, MO) was done as previously described (Theurkauf, 1994). Visualization of Sar1-GFP in fixed tissue was done as above, except all buffers used PBS with 25 μg/ml digitonin instead of Triton X-100.

grk In Situ Hybridization

Whole-mount in situ hybridizations to ovaries were performed as described in Wilhelm et al. (2000). *grk* probe was prepared from linearized *grk* cDNA following the protocol outlined in the DIG RNA labeling kit (Roche, Indianapolis, IN).

RT-PCR from Tral Immunoprecipitates

Immunoprecipitations for RT-PCR were performed as previously described (Wilhelm et al., 2000) with the following modifications. Fifty micrograms of IgG antibody or 50 μl of α-Tral antiserum was added to 125 μl of washed Protein A agarose beads (Invitrogen, Carlsbad, CA) in a final volume of 300 μl and mixed for 30 min at room temperature. One milliliter of hand-dissected ovary extract was added to the beads and allowed to shake for 1 hr at 4°C. The beads were then washed seven times with DXB containing 200 mM KCl, 10 μg/ml leupeptin, pepstatin, and 1 mM PMSF. After elution, DNase treatment, and reprecipitation of the RNA, the RNA sample was resuspended in 30 μl of RNase-free water. One microliter of a 1:100 dilution of the RNA sample was used in the RT-PCR reaction using the One-Step RT-PCR kit (Qiagen, Valencia, CA) with the following program: 50°C 30 min, 95°C 15 min, followed by 25 cycles of 94°C 30 s, 55°C 30 s, 72°C 45 s. Total RNA was isolated by Trizol extraction of 300 μl of extract, and 1 μl of a 1:10 dilution of the RNA sample was used in the RT-PCR reaction. The primers were all designed to span an intron and the sequences are available upon request.

Supplemental Data

Supplemental data include three figures and a movie and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/9/5/675/DC1/.

Acknowledgments

We would like to thank K. Oegema, Y. Zheng, J. White, and C.M. Fan for critically reading the manuscript. We also thank S. Zhou in David King's laboratory for mass spectrometric identification of the Tral complex. J.E.W. is an HHMI fellow of the Life Sciences Research Foundation. M.B. was supported by ACS postdoctoral grant PF-04-022-01-CSM.

Received: April 15, 2005
Revised: August 17, 2005
Accepted: September 16, 2005
Published: October 31, 2005

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